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Hemophilia B in a Female Carrier Due to Skewed Inactivation of the Normal X-Chromosome

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A novel missense mutation (codon 351, GCT (Ala) → CCT (Pro)) of the FIX gene was characterised in a young female with mild hemophilia B. She is heterozygous for the FIX mutation inherited from her carrier mother. Analysis of the methyl-sensitive Hpa II sites at the 5′ end of the hypoxanthine phosphoribosyltransferase gene showed that skewed inactivation of the X chromosome carrying her normal FIX gene accounted for the hemophilia phenotype. Am. J. Hematol. 58:72–76, 1998 © 1998 Wiley-Liss, Inc.

Key words: hemophilia B; FIX gene mutation; skewed inactivation; X chromosome

INTRODUCTION

Hemophilia B is a bleeding diathesis due to deficiency of clotting factor IX (FIX) [1]. Its incidence is approximately half that of hemophilia A, i.e., ~1:50,000 live-born males. Being an X-linked recessive disorder, normally, only males are affected and females are carriers with no clinical manifestation of the disease. However, there had been a few reports of females with hemophilia B [2–7]. In the present study, a young female who carried the FIX defect on one of her X-chromosomes was studied and the phenotypic expression of hemophilia is demonstrated to be due to markedly skewed inactivation of the normal X-chromosome.

MATERIALS AND METHODS

Case Report

A female student (aged 20 years), who was a keen athlete at school, gave a history of easy bruising since childhood, episodic ankle or knee pain and swelling particularly after vigorous exercise in her teens. She presented to the University Health Clinic because of prolonged bleeding after tooth extraction. Menstruation was normal. Investigations at presentation revealed that she has mild FIX deficiency of 0.08 IU/ml (NR = 0.5–1.50), normal FVIIIC of 0.82 IU/ml (NR = 0.5–1.50), prothrombin time 12.4 sec (NR = 9.5–12.5), and a prolonged activated partial thromboplastin time 42.7 sec (NR = 22.0–34.0). Physical examination showed that previous episodes of hemarthrosis have resulted in inflammatory changes in her left ankle and right knee. Besides degenerative changes in the ankles, no other lesion was noted on X ray of the hips and knees. Her parents and seven sibs have no history of bleeding tendency.

DNA Analyses

DNA was extracted from peripheral blood leucocytes of the patient and her parents. One-microgram amounts of the patient's DNA was used for polymerase chain reaction (PCR) amplification of exons a to h of the FIX gene as well as their immediate 5′ and 3′ flanking regions, using primers and conditions as described previously [8]. Each amplified exon fragment was gel-purified and sequenced using T7 DNA polymerase as described [9]. Primary (PCR) primers were used as sequencing primers. The sequenced products were analysed on 8M urea gel. In the case of exon h, an additional reverse primer (nt 31219–31200) was also used to provide an overlapping sequence. The nucleotide numbering corresponds to that reported by Yoshitake et al. [10].

Confirmation of Mutation

The presence of a mutation in exon h was confirmed by digestion of the PCR-amplified exon h fragment of

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the patient and her parents with the restriction enzyme BstN I under conditions recommended by the manufacturer (New England Biolabs, Beverly, MA), followed by analysis on 6% polyacrylamide gel (PAG) in 1 × TBE buffer (10 × TBE = 0.89M Tris-borate, 0.89M boric acid, and 0.02M EDTA) at 150 V for 60 min. The gel was then stained with ethidium bromide and visualised under ultraviolet light.

Karyotype Analysis

Complete karyotype analysis was done using peripheral blood white cells, cultured in the presence of phytohemagglutinin for 3 days to induce mitosis. Standard G banding technique was used [11]. Thirty-three metaphases were examined and karyotype analysis was done with the help of the Cytoscan software (Applied Imaging, Sunderland, UK).

Confirmation of Inactivation of the X-Chromosome

HPRT polymorphism. DNA (15 μg) of the patient, her parents, a normal male subject, and two normal female subjects (as control samples) were double-digested with BamH I and Pvu II at 37°C overnight in 400 μl reaction volume in a buffer consisting of 200 μg/ml BSA, 100 mM NaCl, 100 mM $MgCl_2$, and 10 mM Tris pH 8.0. The digested sample was ethanol precipitated and resuspended in 210 μl of deionised water. Two 100-μl aliquots were made, one digested with Hpa II under conditions recommended by the manufacturer (New England Biolabs) and the other aliquot left undigested. Each sample was again ethanol precipitated before electrophoresis through 1% agarose gel. After electrophoresis, DNA was transferred to Hybon C extra membrane (Amersham, Buckinghamshire, UK).

The filter was pre-hybridised for 4 hr at 41°C in 12 ml of pre-hybridization buffer (50% deionised formamide, 50 mM sodium phosphate buffer pH 7.0, 20 × Denhardt's solution, 3 × SSC, 210 μg/ml denatured salmon sperm DNA, and 150 μg/ml yeast RNA) (1 × SSC = 150 mM sodium chloride, 15 mM sodium citrate). Hybridisation was performed at 41°C overnight in a same volume of hybridisation buffer (which was similar to the pre-hybridisation buffer except for 10 × Denhardt's solution and 10% dextran sulfate) containing 2×10^6 cpm of ^{32}P-labelled probe/ml. The probe used was pHPRT-800 (a plasmid containing part of the 5′ region of hypoxanthine phosphoribosyltransferase gene (HPRT)), which was an 800-bp BamH I/Pst I fragment from a Hpa II/Pst I subclone of pB1.7 [12]. The filter was washed twice at 55°C in 0.1 × SSC, 0.1% sodium dodecyl sulfate (SDS) for 30 min each, followed by one washing at 65°C for 30 min. The air-dried filter was autoradiographed at −70°C for 4 days.

PGK polymorphism. Fifteen micrograms of DNA from the patient, her parents, and the same normal male and normal female control subjects as double digested with Pst I and BstX I under conditions described previously. Half the digested sample was further digested with Hpa II and the other left undigested. The samples were then electrophoresed, Southern transferred, and hybridized to a ^{32}P-labelled phosphoglycerate kinase (PGK) gene probe, which was an 800 bp BamH I/EcoR I fragment from the 5′ end of the PGK gene cloned into the BamH I/EcoR I sites of pSP64 [12]. The conditions for hybridisation and washing were as described for the HPRT polymorphism.

RESULTS

Direct genomic sequencing of PCR-amplified exons of the FIX gene and their immediate 5′ and 3′ splice junctions revealed a single base substitution at exon h, nt 31172 G → C. This corresponds to a missense mutation at codon 351 GCT (Ala) → CCT (Pro) (Fig. 1). Amino acid numbering is according to Anson et al. [13]. The mutation created a BstN I enzyme recognition site. Digestion of the exon h fragment of the patient with this enzyme produced two abnormal smaller fragments of 402 and 253 bp in addition to the normal 655-bp fragment from her normal FIX gene. The propositus' mother gave a similar digestion pattern (Fig. 2). This confirmed that she was also a carrier of the same FIX mutation. Her father showed only the normal size fragment, indicating a normal FIX gene.

Examination of 33 metaphases from a 72-hr culture of phytohemagglutinin-stimulated lymphocytes revealed that all metaphases were normal 46 XX.

Confirmation of Inactivation of the X-Chromosome

Heterozygosity for BamH I/Pvu II restriction fragment length polymorphism (RFLP) of the HPRT gene located on the X chromosome was used to distinguish the maternal and paternal alleles in the patient. Presence or absence of the BamH I RFLP site should yield DNA fragments of 12 and 18 kb, respectively, when hybridised to the pHPRT800 probe. Further digestion of the DNA with the methyl-sensitive Hpa II distinguished active from inactive alleles of the X chromosome. Hpa II digestion converted the active allele from 18 and 12 kb to 17.2 and 11.2 kb, respectively (Fig. 3a). There are two types of inactive pattern. The type I inactive pattern found in the patient, her mother, and the two normal females (control), showed fragments of either 6.8 kb or 8 and 6.8 kb after Hpa II digestion. The type II inactive pattern found in the patient and the two normal females showed unchanged size of the 12-kb fragment (Fig. 3a and b). In

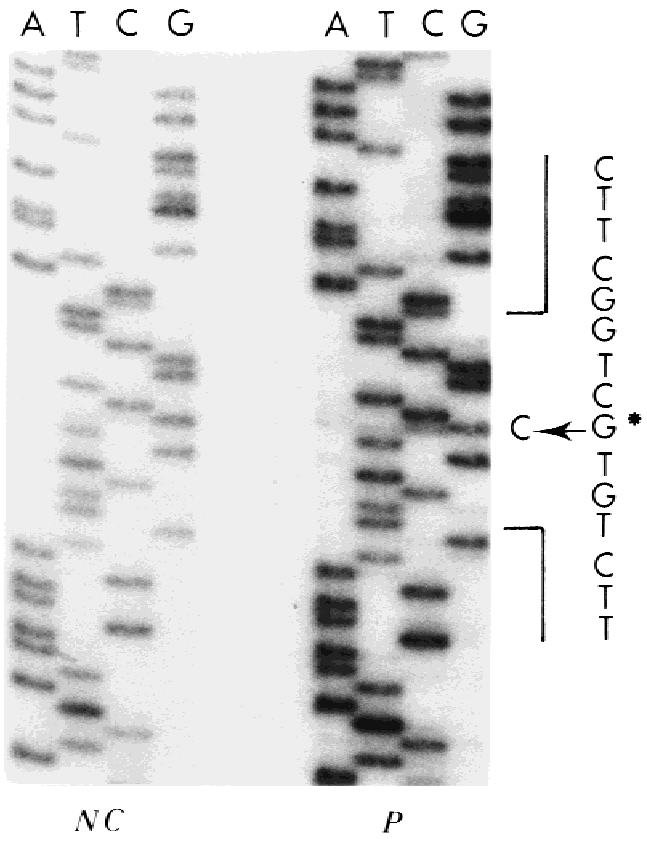

Fig. 1. Direct genomic sequencing of PCR-amplified exon h of the FIX gene of the patient (P) compared to that of a normal male subject (NC). *The position of the missense mutation in one allele (G → C). The sequence is shown in the forward strand. Since the propositus is a female, her normal FIX gene fragment sequenced at the same time showed G at the same position.

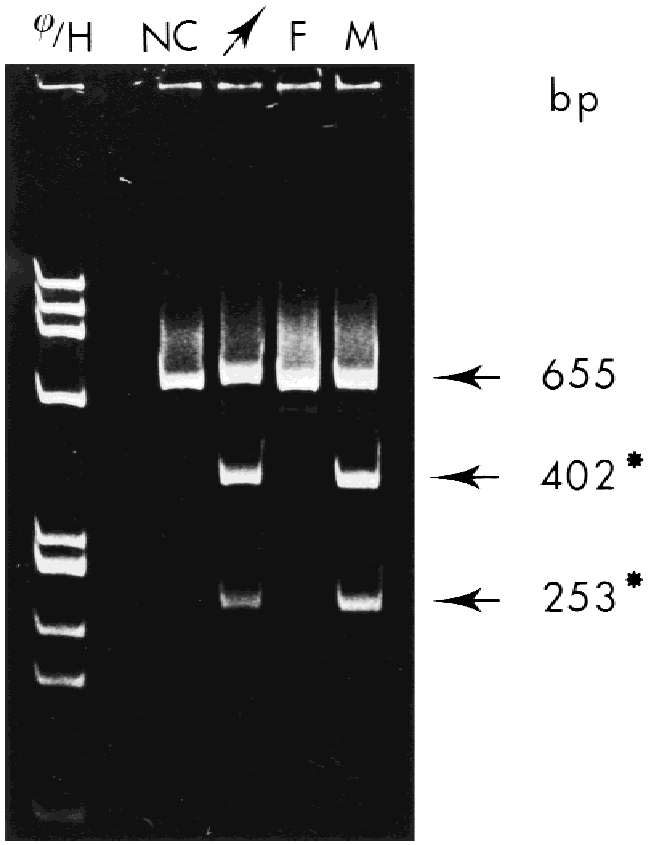

Fig. 2. Polyacrylamide gel electrophoresis of the BstN I-digested amplified FIX gene exon h fragment (655 bp) in the patient (↗), her father (F) and mother (M) compared to that in a normal control (NC) subject. The mutation created a new enzyme recognition site and resulted in two smaller fragments (*) of 402 and 253 bp.

Figure 3b, after Hpa II digestion (+ lane) of the DNA from the propositus, the 18-kb fragment was all converted to 17.2 kb (active) while the 12-kb fragment remained either the same size (inactive, type II) or converted to 6.8 kb (inactive type I) and there was no 11.2-kb (active) fragment (lane 5).

Homozygosity for BstX I polymorphic site at the 5′ end of the PGK gene was found in the patient. Hence, it was impossible to distinguish the paternal from the maternal X chromosome using this approach.

DISCUSSION

Hemophilia A or B in females is a rare occurrence. The possible causes of clinical hemophilia in females have been documented to be (1) due to homozygosity for the hemophilia gene resulting from consanguinity [2,14] or double de-novo mutations [15], (2) deletion (Turner's syndrome [16]) or structural abnormalities of the X-chromosomes [3,4,17], (3) non-random skewed inactivation of the normal X-chromosome [6,7], or (4) theoretically, due to genetic homozygosity or uniparental disomy.

In this study, both the propositus and her mother have one normal FIX gene and one mutant gene. The mother was only a carrier with FIX level of 0.58 IU/ml and she did not have any bleeding tendency. Hence both homozygosity of the hemophiliac gene and uniparental disomy can be ruled out. Cytogenetic analysis of the propositus showed a normal karyotype, 46 XX, with no deletion, inversion, or gross structural abnormality of the X chromosomes. Thus, the only possible mechanism for manifestation of disease in the propositus was skewed inactivation of the X chromosome carrying the normal FIX gene, which, in this patient, is paternal in origin. It was possible to demonstrate this by analysis of X chromosome inactivation based on methylation of Hpa II sites at the 5′ end of the HPRT gene [12]. Since the mother was homozygous for the 18-kb BamH I/Pvu II fragment of the HPRT gene, the 12-kb fragment of the propositus must be paternal in origin. In the propositus, the 12-kb

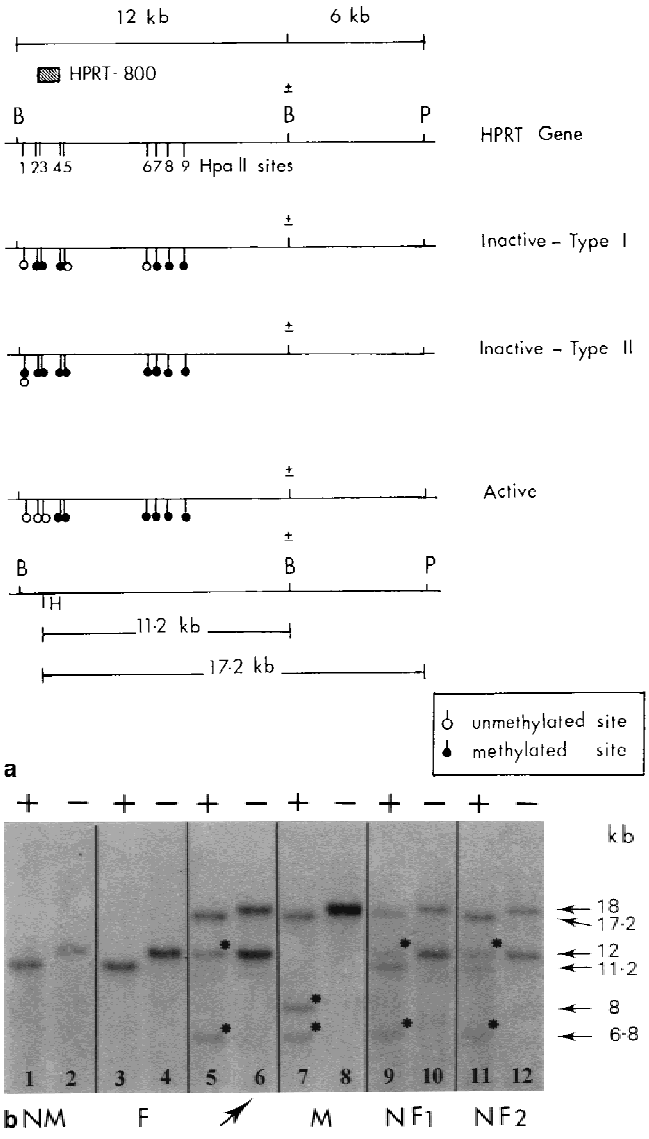

Fig. 3. a: A restriction map of the 5′ region of the HPRT gene showing the 5′ Bam HI (B) site on the left through to the Pvu II (P) site on the right. Nine Hpa II (H, 1–9) sites and a polymorphic Bam HI (B ±) site lie in between. The fragment sizes, 12 and 18 kb are recognizable by hybridisation to probe HPRT-800 (shaded box). Hpa II site 1 is unmethylated (♘) in active X-chromosomes and in some inactive X-chromosomes. Hpa II sites 2–9 are differentially methylated (●). In active alleles, Hpa II sites 2 and 3 are unmethylated (cut by enzyme), while sites 4 to 9 are all methylated (uncut by enzyme). In type I inactive alleles, Hpa II sites 2 and 3 are methylated, while at least one of sites 4 to 9 is unmethylated, generating varying sized shorter fragments upon digestion with the enzyme (see b, lanes 5, 7). In type II inactive alleles, Hpa II sites 2 to 9 are all methylated, hence no alteration in fragment size after enzyme digestion (Fig. 3b, lane 5). b: Southern blot of BamH I/Pvu II digested genomic DNA showing a polymorphic region at the 5′ end of the HPRT gene (− lanes). The two alleles were 18 and 12 kb, respectively. Further digestion with Hpa II converted the active X chromosomes to 17.2 and 11.2 kb, respectively (+ lanes). Hpa II digestion of type I inactive alleles resulted in fragments of 6.8 kb or 8 and 6.8 kb, respectively, in the propositus (♐), her mother (M), and two normal females (NF_1 and NF_2) (* in (+) lanes 5, 7, 9, and 11). Hpa II digestion of type II inactive allele did not result in any change of fragment size (12-kb fragment * in (+) lanes 5, 9, and 11) in the propositus and normal females. F = father, NM = normal male.

fragment was shown to be inactive as represented by the unchanged fragment size after digestion with Hpa II (Type II inactive) and no active fragment of 11.2 kb was seen, as in the two normal females. This indicated an extreme bias in favour of inactivity of the paternal X chromosome in the leucocytes and probably in the liver cells that produce FIX.

There had been a previous report of severe hemophilia in a female where preferential inactivation of the normal X chromosome was due to minor chromosome deletion [5] or coinheritance of a lethal gene [18]. Also, in a study of X-linked disease in monozygotic twins, opposite skewing in X-inactivation pattern is known to occur, where the abnormal allele is inactivated in most cells of the normal twin and the normal allele inactivated in most cells of the affected twin, thereby giving rise to disease in the latter [19]. However, the reason for the skewed inactivation of the normal X chromosome in most other reported cases and in this patient is unknown.

The FIX mutation of the propositus was a missense mutation at codon 351, exon h, which lies in the catalytic domain. This involved a change of amino acid from alanine to proline. According to Chou and Fassman's [20] prediction of the protein structure, it causes an increase turn in the molecule with decreased hydrophilicity. A male offspring of hers inheriting the mutation would, of course, manifest hemophilia B phenotype. However, male offsprings bearing the normal X chromosome might have inherited a yet undetermined lethal genetic lesion although no such evidence is present in males of the family.

The present case emphasizes that X-linked inheritance of coagulation disorders may manifest in rare instances in females and awareness of such occurrences, with

prompt treatment when necessary, will avoid joint deformities in the affected.

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REFERENCES

1. Thompson AR: Structure, function and molecular defects of factor IX. Blood 67:565, 1986.
2. Morita H, Kagami M, Ebata Y, Yoshimura H: The occurrence of homozygous hemophilia in the female. Acta Haematol (Basel) 45:112, 1971.
3. Spinelli A, Schmid W, Straub PW: Christmas disease (haemophilia B) in a girl with deletion of the short arm of one X-chromosome. Br J Haematol 34:129, 1976.
4. Lusher JM, McMillan CW, the Hemophilia Study Group: Severe factor VIII and factor IX deficiency in females. Am J Med 65:637, 1978.
5. Nisen P, Stamberg J, Ehrenpreis R, Velasco S, Sheude A, Engelberg J, Karayalcin G, Waber L: The molecular basis of severe hemophilia B in a girl. New Engl J Med 315:1139, 1986.
6. King S, Coffey KS, Ljung AR, Sjorin E, Nilsson IM, Holmberg L, Giannelli F: Moderate haemophilia B in a female carrier caused by preferential inactivation of the paternal X chromosome. Eur J Haematol 47:257, 1991.
7. Palmer S, Standen GR, Yates P, Oakhill A: Genetic analysis in a female manifesting haemophilia B. Postgrad Med J 70:828, 1994.
8. Chan V, Yip B, Tong TMF, Chan TPT, Lau K, Yam I, Chan TK: Molecular defects in haemophilia B: Detection by direct restriction enzyme analysis. Br J Haematol 79:63, 1991.
9. Chan V, Chan TK, Kan YW, Todd D: A novel β-thalassemia frameshift mutation (codon 14/15), detectable by direct visualization of abnormal restriction fragment in amplified genomic DNA. Blood 72: 1420, 1988.
10. Yoshitake S, Schach BG, Foster DC, Davie EW, Kurachi K: Nucleotide sequence of the gene for human factor IX (antihemophilic factor B). Biochemistry 24:3736, 1985.
11. Verma RS, Babu A: Manual of basic techniques. In: ''Human Chromosomes.'' New York: Pergamon Press, 1989, pp 47–49.
12. Vogelstein B, Fearon ER, Hamilton SR, Preisinger AC, Willard HF, Michelson AM, Riggs AD, Orkin SH: Clonal analysis using recombinant DNA probes from the X-chromosome. Cancer Res 47:4806, 1987.
13. Anson DS, Choo KH, Rees DJG, Giannelli F, Gould K, Huddleston JA, Brownlee GG: The gene structure of human anti-haemophilic factor IX. EMBO J 3:1053, 1984.
14. Pietu G, Thomas-Maison N, Sie P, Larrieu MJ, Myer D: Haemophilia A in a female: Study of a family using intragenic and extragenic restriction site polymorphisms. Thromb Haemost 60:102, 1988.
15. Windsor S, Lyng A, Taylor SAH, Ewenstein BM, Neufeld EJ, Lillicrap D: Severe haemophilia A in a female resulting from two de novo factor VIII mutations. Br J Haematol 90:906, 1995.
16. Bithell TC, Pizarro A, MacDiarmid WD: Variant of factor IX deficiency in female with 45, X Turner's syndrome. Blood 36:169, 1970.
17. Gilgenkrantz S, Briquel ME, Mandel JL, Oberle I: A case of female hemophilia with a 46, XXr karyotype studied with X-chromosome DNA probes. Hum Genet 72:157, 1986.
18. Coleman R, Genet SA, Harper JI, Wikie AOM: Interaction of incontinentia pigmenti and factor VIII mutations in a female with biased X inactivation, resulting in haemophilia. J Med Genet 30:497, 1993.
19. Tiberio G: MZ female twins discordant for X-linked diseases: A review. Acta Genet Med Gemellol 43:207, 1994.
20. Chou P-Y, Fassman GD: Prediction of the secondary structure of proteins from their amino acid sequence. Adv Enzymol 47:45, 1978.